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PATENT
0020-4546P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Toru KIMURA *et al.*

Serial No.: 09/284,180 Group: 1633

Filed: June 9, 1999 Examiner: S. Chen

For: NOVEL SEMAPHORIN : SEMAPHORIN W

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I, Kaoru KIKUCHI, residing at 2-11-15-909, Minamihibarigaoka, Takarazuka-shi, Hyogo, JAPAN, declare as follows:

1. I am a citizen of JAPAN.

2. I am currently employed in research activities at Sumitomo Pharmaceuticals Company, Limited. I am engaged in research on Semaphorin, and am responsible for the cloning of Semaphorin Y and Semaphorin Z, in addition to Semaphorin W. I am an author or co-author of the following Semaphorin-related papers:

"Molecular cloning of a novel member of semaphorin family genes, semaphorin Z", Kikuchi K, Ishida H, Kimura T., *Mol. Brain Res.* **51** (1997) 229-37; "Cloning and characterization of a novel class VI semaphorin,

semaphorin Y", Kikuchi K, Chedotal A, Hanafusa H, Ujimasa Y, de Castro F, Goodman CS, Kimura T., *Mol Cell Neurosci* **13** (1999) 9-23; and "Cloning, expression, and genetic mapping of Sema W, a member of the semaphorin family", Encinas JA, Kikuchi K, Chedotal A, de Castro F, Goodman CS, Kimura T., *Proc. Natl. Acad. Sci.* U.S.A. **96** (1999), 2491-2496

3. I have read and understand the subject matter of U.S. Application Serial Number 09/284,180 and am familiar with the prosecution of the application.

4. I have carried out, or supervised, computer search of sequence data available from various databanks. The computer search was conducted as follows:

About 1,600,000 kinds of nucleotide sequences deposited at mainly GenBank, EMBL, DDBJ and PDB were searched for the presence of a DNA homologous to rat semaphorin W identified in SEQ ID NO: 2 with a computer software: BLASTN (default). It is known that nucleotide sequences hit in the search for a homologue of a given DNA using said software "BLASTN" have 80% or more identity to said DNA in question.

The sequences hit were evaluated regarding the relevance to Semaphorin W according to the Alignment sore, Bit score and E-value.

The results are shown in Figure 1 hereto attached. Fig. 1 shows that there are 58 kinds of sequence having at least 80% identity to SEQ ID NO: 2 in the database

searched (one line in Fig. 1 corresponds to one sequence). It is also shown that the sequences relevant to Semaphorin W (depicted in pink or red line), show homology to Semaphorin W extensively. On the other hand, DNAs irrelevant to Semaphorin W (green or blue line) show homology to only a small partial region (less than 30 base pairs) of Semaphorin W.

In conclusion, the computer search revealed that DNAs homologous to a full or sufficient length of SEQ ID NO: 2 are all related to Semaphorin W.

5. I hereby declare that all statements made herein of my own knowledge are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 10 day of February, 2003.

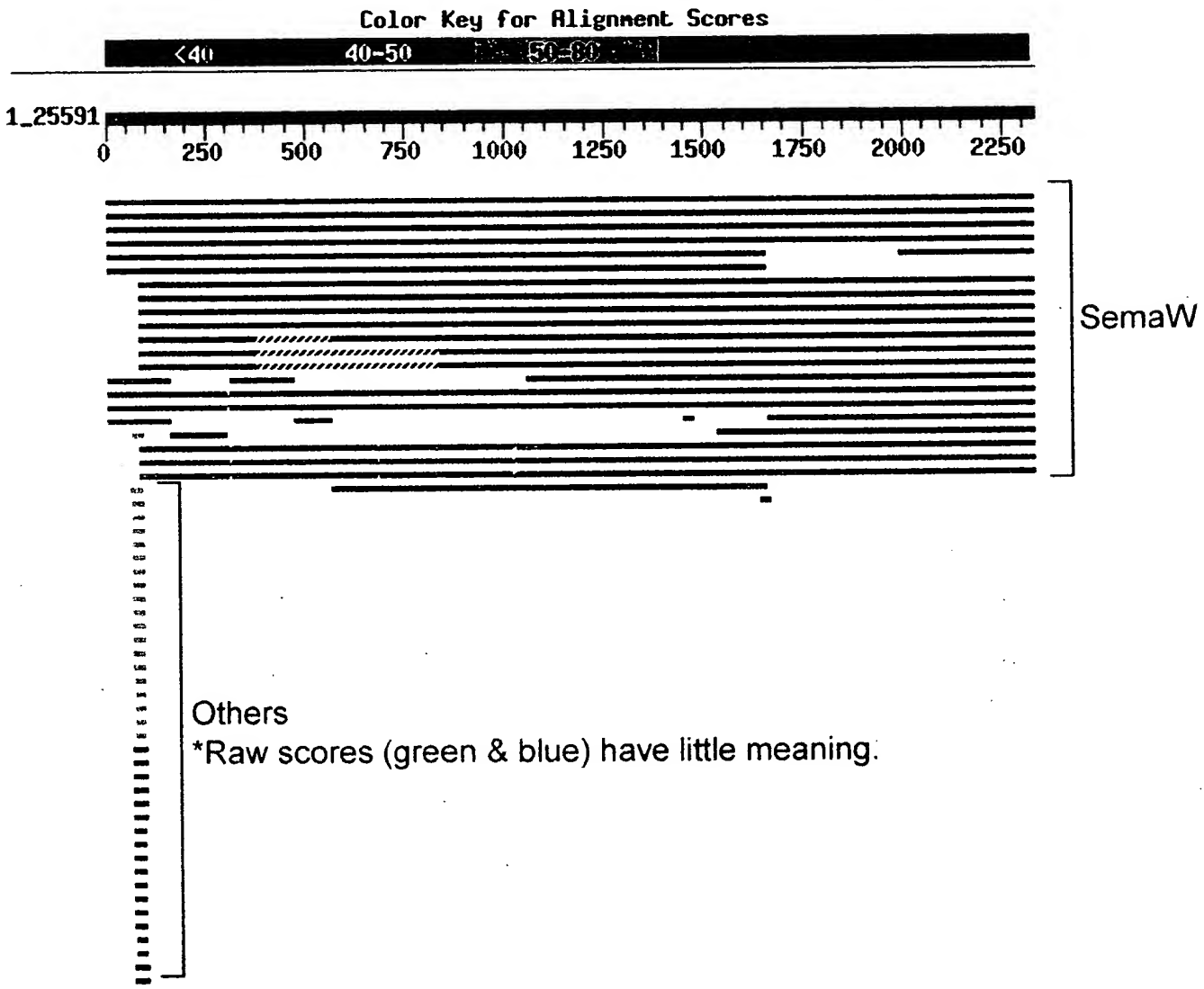
Kaoru Kikuchi

Kaoru Kikuchi, Ph.D.

BLASTN 2.2.5 [Nov-16-2002]

Query=
(2331 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)
1,672,710 sequences; 7,833,321,048 total letters



*Raw scores (green & blue) have little meaning.

Cell, Vol. 75, 1389–1399, December 3

The *semaphorin* Genes Encode a Family of Transmembrane and Secreted Growth Cone Guidance Molecules

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Summary

In addition to its expression on subsets of axons, grasshopper Semaphorin I (Sema I, previously called Fasciclin [Fas] IV) is expressed on an epithelial stripe in the limb bud, where it functions in the guidance of two sensory growth cones as they abruptly turn upon encountering this sema I boundary. We report here on the cloning and characterization of two *sema* genes in Drosophila, one in human, and the identification of two related viral sequences, all of which encode proteins with conserved Semaphorin domains. Drosophila sema (D-Sema) I is a transmembrane protein, while D-Sema II and human Sema III are putative secreted proteins that are similar to the recently reported chick collapsin. D-Sema I and D-Sema II are expressed by subsets of neurons and muscles. Genetic analysis in Drosophila reveals that *semaII* is an essential gene that is required for both proper adult behavior and survival.

Introduction

Growth cones make specific pathway choices and ultimately find their correct targets using a variety of different types of guidance cues (reviewed by Goodman and Shatz, 1993). While much is known about the ligands and receptors that control cell and substrate adhesion, less is known about the molecules that control some of the other major guidance forces, such as chemoattraction (e.g., Lumsden and Davies, 1986; Tessier-Lavigne et al., 1988; Heffner et al., 1990) and both contact-mediated (Kapfhammer et al., 1986; Kapfhammer and Raper, 1987a, b) and apparently diffusible (Pini, 1993) repulsive signals. Evidence is also starting to accumulate that suggests certain regions (for example, midline structures) can provide simultaneous attractive and repulsive signals to different growth cones (Godement et al., 1990; Sretavan, 1990; Sretavan and Reichardt, 1993; Bernhardt et al., 1992; Seeger et al., 1992).

Members of our laboratory previously conducted a series of monoclonal antibody screens that led to the cloning of the genes encoding five surface glycoproteins expressed on subsets of developing insect central nervous system (CNS) axon pathways. Four of these genes encode homophilic cell adhesion molecules (Patel et al., 1987; Zinn et al., 1988; Harrelson and Goodman, 1988; Snow et al., 1989; Bieber et al., 1989; Grenningloh et al, 1990, 1991). The fifth gene encodes an axonal glycoprotein, initially called grasshopper Fasciclin (Fas) IV, that like the others is also expressed on a subset of axon pathways in the insect embryo (Kolodkin et al., 1992). However, Fas IV differs from the other four in a number of respects: it cannot mediate cell aggregation in vitro (suggesting that it is not a homophilic cell adhesion molecule), it is expressed as a guidance cue in the environment of the growth cone but is not expressed on the responding growth cone, and its sequence, when published in 1992, was novel. In addition to its expression on a subset of CNS axon pathways, Fas IV is also expressed on a stripe of epithelial cells in the embryonic limb bud, where it functions to help guide two sensory growth cones as th y sharply turn upon encountering this Fas IV stripe (Figur 1; see Discussion) (Kolodkin et al., 1992).

Given the discovery reported here of a gene family encoding related transmembrane and secreted proteins and the differences between *fasIV* and the other *fas* genes, all of which encode homophilic cell adhesion molecules, it seemed appropriate to rename this protein and the gene family to which it belongs. We call this initial grasshopper protein Semaphorin I (G-Sema I), and the family the Semaphorins, to denote their function as semaphores (or signals) for growth cone guidance.

In the present study, we have used a polymerase chain reaction–based (PCR-based) approach to clone two diff r-ent *sema* genes in Drosophila and a related *sema* gene in humans. Based on the conserved amino acids in these sequences, we have also identified related sequences in two poxviruses (vaccinia [closely related to the cowpox] and variola [human smallpox]), suggesting potential functions for Semaphorins in the immune system. While our work was being prepared for publication, the sequence of chick collapsin was published (Luo et al., 1993). Collapsin is highly related to G-Sema I (Kolodkin et al., 1992) and the other Semaphorins described here.

The two different Drosophila Semaphorins are expressed by different subsets of neurons and muscles. G - netic analysis of *D-semaII* reveals that it is an essential gene required for both proper adult behavior and survival. In the absence of Sema II, many mutant flies do not eclose as adults. Those that do eclose cannot fly, most show abnormal visual orientation, many do not drink, and all di within a few days.

Results

Identification of a Family of *sema* Genes in Drosophila

The grasshopper *semaI* gene (formally *fasIV*) encodes the transmembrane protein G-Sema I that functions in growth cone guidance (Figure 1) (Kolodkin et al., 1992) (see Discussion). Given this function, we undertook a search for its homolog in Drosophila. Such a large evolutionary jump might be difficult because these two ins ct species (the grasshopper Schistocerca americana and the fruitfly Dro-

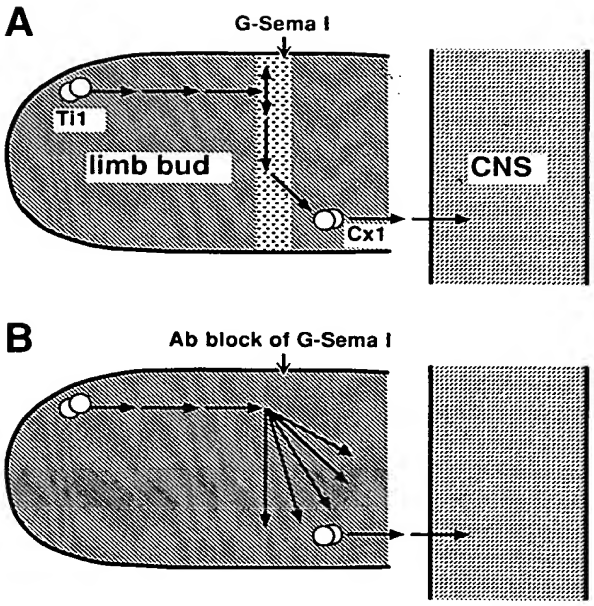
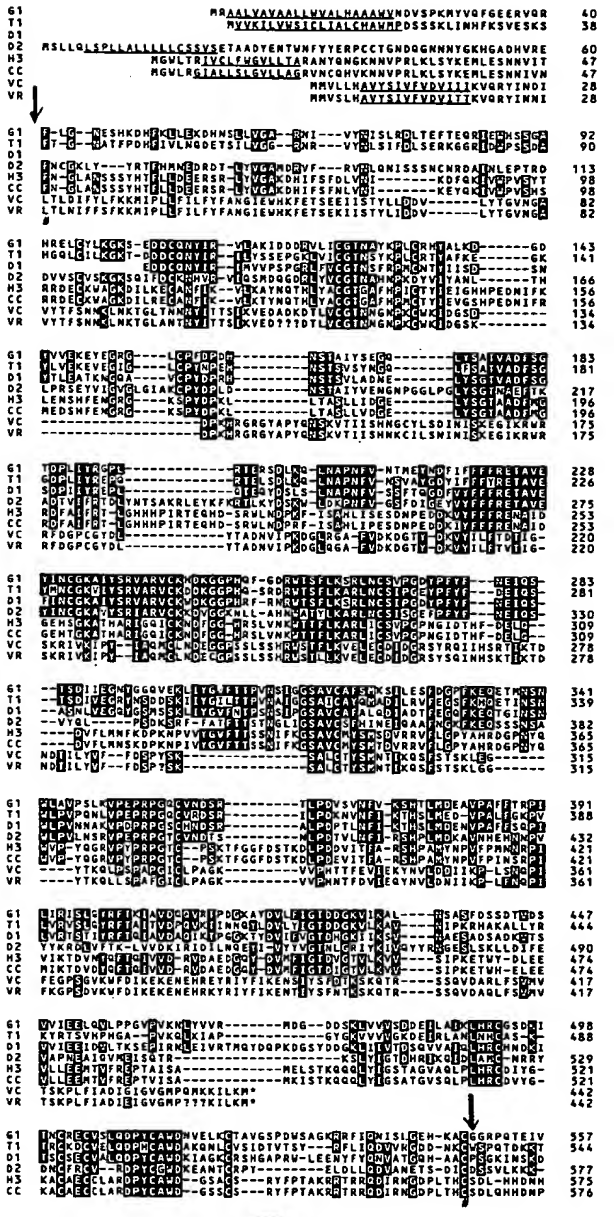


Figure 1. Expression and Function of Sema I in the Grasshopper Limb Bud

Schematic diagrams of 35% grasshopper limb buds showing (A) the trajectory of the pair of Ti1 growth cones in relationship to the epithelial expression of Sema I as they pioneer an axon pathway towards the CNS, and (B) the aberrant trajectory of these growth cones following application of antibodies that bind Sema I (Kolodkin et al., 1992).

(A) The long dark arrows represent the sequential steps in the pathfinding by the Ti1 growth cones (O'Connor et al., 1990). They first extend proximally towards the CNS, then stop and reorient once they encounter the Sema I boundary (initially extending in both the dorsal and ventral directions), then extend ventrally as a tightly fasciculated pair of axons along the distal portion of Sema I–expressing cells, reorient as they turn proximally after filopodial contact with the Cx1 neurons, and finally extend into the CNS.

(B) In cultured limb buds in which antibodies against Sema I were added during the period of axon outgrowth, the Ti1 growth cones often extend across the Sema I–expressing stripe, ultimately turning ventrally towards the Cx1 neurons, but doing so in a more variable fashion. The growth cones branched into multiple axons that were highly defasciculated in this location. See Discussion.



sophila melanogaster) diverged some 300 million years ago. Moreover, since G-Sema I was a pioneer protein with no similarity to other proteins and no internal repeats, it was difficult to predict which residues might be conserved across species.

A PCR-based evolutionary approach addressed this problem, incorporating the use of degenerate primers designed from several G-Sema I s quences that we guessed might b of structural or functional significance. One pair of primers (see Experimental Procedures) was used in low stringency PCR protocols with genomic DNA templates to isolate related sequences from a beetle, Tribolium confusum, and from a moth, Manduca sexta. The PCR product from Tribolium was used to isolate cDNA clones. The deduc d amino acid sequence for the Tribolium open reading frame (ORF) is shown in Figure 2. This sequence

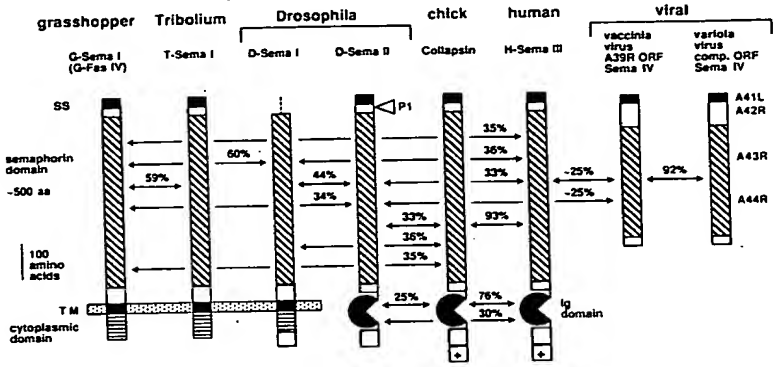


Figure 3. Schematic Diagram of Semaphorin Proteins

Schematic representations of G-Sema I (Kolodkin et al., 1992), the other three insect, one human, and two viral Sema proteins described here and chick collapsin (Luo et al., 1993). These proteins begin with a signal sequence (SS) and are thus likely to encode transmembrane or secreted proteins. All six eukaryotic sema share a 500 amino acid Sema domain (hatched boxes). The two viral sequences encode truncated and more divergent Sema domains. The percentages along the arrows denote the percent amino acid identity between the aligned domains in the pairs of sequences. Three of the insect proteins appear to be homologs (G-Sema I, T-Sema I, and D-Sema I); all three have a transmembrane domain (TM) followed by a modestly conserved cytoplasmic domain (horizontal stripes). D-Sema I has an additional 32 amino acids at the C-terminus of its cytoplasmic domain (open box) not found in the other two (the N-terminal sequence of D-Sema I is incomplete). D-Sema II, chick collapsin, and H-Sema III have neither transmembrane domains nor any other potential membrane linkage, but rather have a single Ig domain (closed semicircle) followed by another short stretch of amino acids (open box). Chick collapsin and H-Sema III have an additional 45 amino acids at their C-terminus that include 18–19 (respectively) positively charged amino acids; this region is shown as a basic (plus sign) domain. The vaccinia virus Sema sequence is found in ORF A39R. The variola virus Sema sequence was compiled from the nucleotide sequences in and around ORFs A41L, A42R, A43R, and A44R. D-Sema II has a triangle labeled P1 that denotes the location of a P element inserted in the codon for amino acid 32 that leads to the loss-of-function mutation *semaII*^{P1}.

information was then used to refine our primer design for PCR reactions using Drosophila embryonic cDNA.

Three pairs of primers (see Experimental Procedures) were used successfully to amplify Drosophila sequences related to G-Sema I. Two pairs of primers (that shared a common 3′ primer) gave products encoding G-Sema I–related sequences that are similar but not identical. Subsequent analysis confirmed the identification of two different Drosophila *sema* genes. The two different PCR products were used to isolate two different classes of cDNA clones. The two Drosophila ORFs are shown in Figur 2, and the protein structures are shown schematically in Figure 3.

The Tribolium protein and one of the Drosophila proteins appear to be homologs of the G-Sema I protein because they share 60% amino acid identity with G-Sema I along its entire length (see Figure 2; Figure 3). Thus we call these proteins Tribolium Sema (T-Sema) I and D-Sema I. The other Drosophila protein has a lower degree of amino acid identity and a distinct C-terminal structure, defining a second protein called D-Sema II.

The two new complete insect sequences (T-Sema I and D-Sema II) encode proteins with signal sequences (von Heijne, 1986), as does G-Sema I. T-Sema I and D-Sema I have transmembrane domains (Chou and Fasman, 1974), as does G-Sema I. However, D-Sema II has no transmembrane domain, its C-terminus shows no indication of a potential phospholipid linkage, and thus it is likely to be secreted. In addition, at its C-terminus, D-Sema II has a single C2-type immunoglobulin (Ig) domain (Williams and Barclay, 1988) (see Figure 2; Figure 3).

All four insect Semaphorins share a highly conserved extracellular domain of 500 amino acids (using basic local alignment search tool P. Altschul et al., 1990; the PIR data base) that is characterized by 16 conserved cysteines, one conserved potential N-linked glycosylation site, and numerous blocks of conserved amino acids throughout the 500 amino acid domain (see Figure 2). In their Semaphorin domains (comprising two-thirds of each protein), the three putative homologs (G-Sema I, T-Sema I, and D-Sema I) are most similar and all are more divergent from D-Sema II in t rms of both percent identity and the absence of several blocks of amino acids found in D-S ma II. The existence of two different *sema* genes in the same species (Drosophila) suggests that they define a novel gene family encoding both transmembrane and secreted proteins.

Figure 2. Amino Acid Alignments of Semaphorin Proteins

Alignments of G-Sema I (G1) (Kolodkin et al., 1992), the other three insect (T1, D1, D2), one human (H3), and two viral (VC, VR) Sema amino acid sequences presented in this study, and chick collapsin (CC) (Luo et al., 1993), are shown. All of these proteins share a highly conserved 500 amino acid domain (delimited by the number symbols), that defines the Sema domain. Tribolium Sema I (T1) and D-sema I (D1) appear to encode homologs of G-sema I in that all three are highly conserved transmembrane proteins (the sequence for D-Sema I is incomplete in its N-terminal end). In contrast, D-Sema II (D2), H-Sema III (H3), and chick collapsin encode proteins that lack a transmembrane domain, have a single immunoglobulin domain, and have no apparent membrane linkage. H-Sema III and chick collapsin, but not D-Sema II, end with a highly basic domain. The two viral sequences (vaccinia virus A39R ORF [VC] and variola virus composite ORF [VR]) encode proteins with truncated and more divergent Sema domains (question mark denotes residues in the variola sequence that are ambiguous at points of likely frame shifts; see Results). In the Sema domain, we define conserved residues by their presence in at least three protein sequences, only one of which can be viral (owing to their 92% amino acid identity) and only one of which can be collapsin or H-Sema III (owing to their 93% amino acid identity). In the transmembrane and cytoplasmic domains, we define conserved residues by their presence in at least two of the three protein sequences. Black outline, conserved residues; underline, signal sequence; carets, transmembrane domain; double dashes, immunoglobulin domain; and asterisks, stop codons. The four insect proteins share a common potential N-linked glycosylation site (at amino acid 163 in G-Sema I); the two vertebrate proteins have different shared sites.

Identification of a Human *semaphorin* Gene

Based on the insect sequences, we used PCR to isolate related human genes. One pair of primers was used successfully to amplify a product using as a template DNA from a human fetal brain cDNA library. This product was used to screen this library to isolate cDNA clones. Translation of the human ORF (see Figure 2) reveals a deduced protein with a signal sequence, a large extracellular domain, and no transmembrane domain. Human Sema (H-Sema) III contains a Semaphorin domain that is highly similar to the Sema domains in the four insect proteins. H-Sema III contains all but 2 of the 16 conserved cysteines characteristic of the insect Sema domains. At its C-terminus, H-Sema III contains a single C2-type Ig domain, making it most like D-Sema II in overall structure.

However, the amino acid sequence of the Sema domain in H-Sema III is equally divergent from the two Drosophila proteins (36% with D-Sema I and 33% with D-Sema II; see Figure 2; Figure 3), and thus we have given it a different designation. Although structurally similar to D-Sema II, the H-Sema III sequence diverges at its C-terminal end, where its last 45 amino acids include 19 positively charged residues forming a basic domain.

Identification of Poxvirus Sequences Encoding Secreted Semaphorins

Upon searching the PIR data base with all five Sema sequences, we found a modest but significant amino acid similarity with the sequences of several ORFs encoded by two members of the pox family of viruses: vaccinia virus, a close relative of cowpox virus, and variola virus, the human smallpox virus. The vaccinia virus Sema sequence is from ORF A39R (Amegodzie et al., 1992) (see Figure 2; Figure 3) and contains a putative signal sequ nce and a truncated Sema domain of 375 amino acids that aligns along its length with the other Sema domains, including 6 of the 10 conserved cysteines in this region and with 25% amino acid identity. The vaccinia Sema IV protein has no transmembrane domain, no other potential membrane linkage, and no Ig domain. Thus it is likely to encode a secreted protein consisting almost entirely of a truncated Sema domain.

The variola virus sequence was less obvious because w had to assemble it as a composite based on the contiguous nucleotide sequences in and around the reported ORFs A41L, A42R, A43R, and A44R (Shchelkunov et al., 1993) (see Experimental Procedures). The composite variola ORF is 92% identical at the amino acid level to the vaccinia Semaphorin over its entirety, with no deletions or additions (see Figure 2). The two viral Semaphorins show only modest amino acid identity to the other Semaphorins (25%) and have several regions of additions or deletions. Nevertheless, these conserved amino acid blocks and spacing are highly significant.

Drosophila Semaph rins Are Expressed by Neur ns and Muscles

To determine the pattern of expression of the two fly Semaphorins, we used digoxigenin-labeled riboprobes for in situ hybridization to localize their RNA in whole-mount embryos. *sema I* expression begins around stage 10, and is seen primarily in the developing CNS. In stage 16 embryos, *sema I* is expressed at its highest levels throughout the CNS (Figures 4A and 4B). Most CNS neurons appear to express *sema I* at this stage. In addition, weak expression is seen in portions of the peripheral nervous system, most clearly in the lateral sensory clusters (Figure 4A). Therefore, like *G-sema I*, *D-sema I* is expressed in the CNS. However, *D-sema I* appears to be expressed by a significantly larger number of neurons than is *G-sema I*.

sema II expression also begins around stage 10 in weak epidermal stripes. Nervous system expression is first detected around stage 15 and at stage 16 appears to remain restricted to a smaller subset of neurons than *sema I* (Figures 4C and 4D). Beginning around stage 14, *sema II* is also expressed at a very high level in a single ventral thoracic muscle fiber in segment T3 (Figures 4C–4E; Figure 5). In addition, an enhancer trap P element (*sema II^{P2}*; see next section) inserted in the *sema II* locus expresses β-galactosidase (β-gal, which in this construct carries a nuclear localization signal) in a pattern identical to that seen by in situ analysis, including localization in the nuclei of this single muscle fiber (see Figure 4F). *sema II* is also expressed in the embryonic gonads (see Figure 4C) and in anterior sensory organs, including the maxillary complex.

D-sema II Is an Essential Gene Required for Adult Behavior and Survival

sema I is located at 29E1–2 and *sema II* at 53C9–10 on the second chromosome of Drosophila. Several lethal P element insertions have been localized to the 53C region. Genetic crosses show that the P element designated 3021 (here called *sema II^{P1}*) and the P element designated 3474 (here called *sema II^{P2}*) fail to complement one another and thus might be in the same gene. Polytene chromosome in situ hybridization confirms that each of these lines contains only a single P element.

We used inverse PCR (Ochman et al., 1988) and standard PCR to isolate genomic DNA sequences adjacent to the insertion sites. The P element in *sema II^{P1}* is inserted in the middle of the codon for amino acid 32 in the ORF. This large transposon is located just after the signal sequence and thus, given its size and many stop codons in all frames, is likely to result in a complete loss-of-function mutation in the gene. The P element in *sema II^{P2}* is inserted about 600 bp upstream of the beginning of our cDNA clone. The *sema II* mRNA is about 4 kb, and yet our cDNA clone is only 2.4 kb with 267 bp of 5′ untranslated sequence. Thus, the *sema II^{P2}* insert is located either in 5′ untranslated or control sequences very clos to the start of transcription or in a 5′ intron.

Both of these P elements in the *sema II* gene are enhancer trap elements containing the *lacZ* gene, which is driven by a neutral promoter that often leads to the expression of β-gal in a pattern that reflects part or all of the expression of the gene into which the P elements have inserted. The pattern of β-gal expression in *sema II^{P2}* is identical to the pattern of RNA expression seen by in situ analysis (see Figures 4E and 4F). The β-gal expression

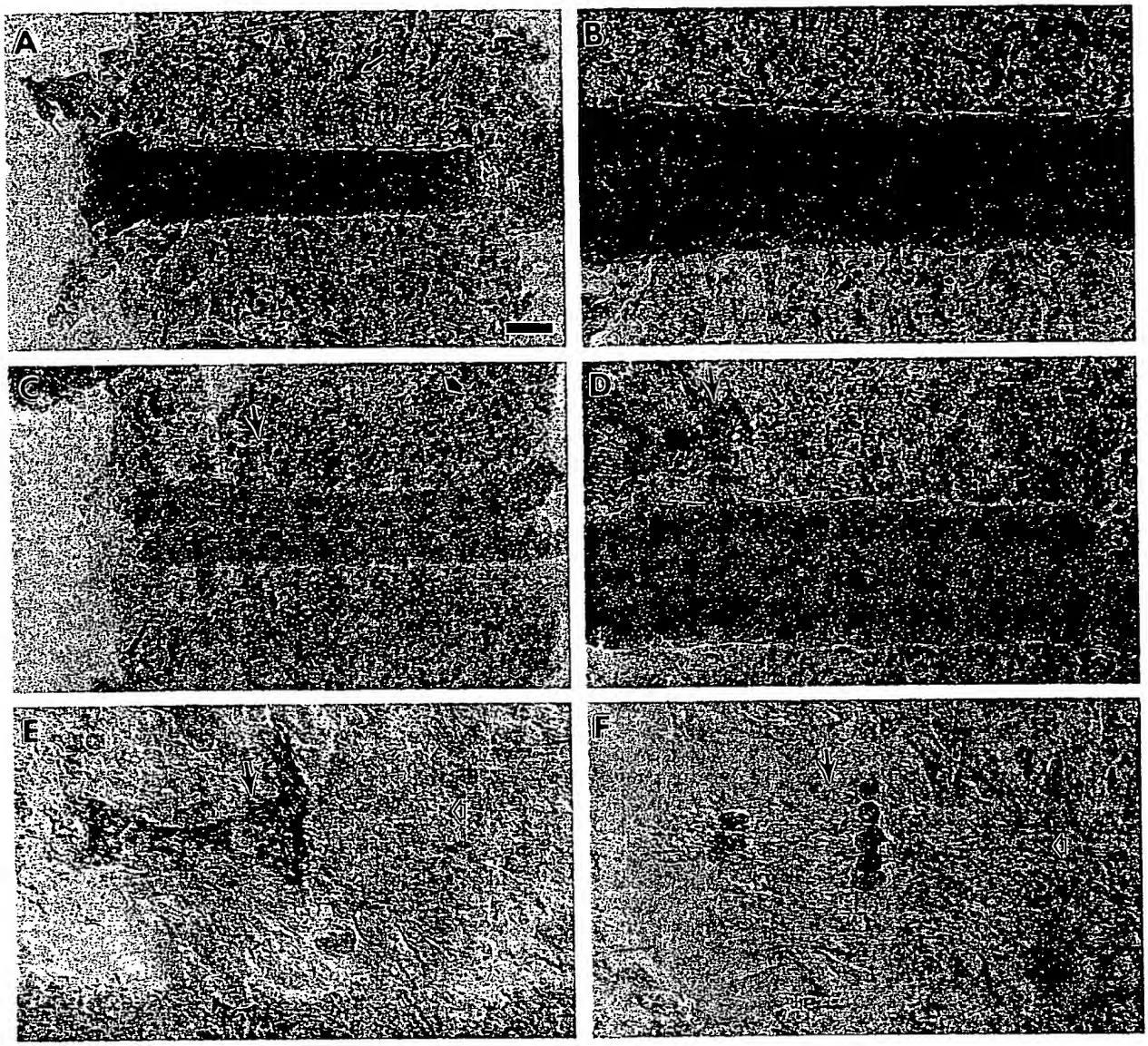


Figure 4. Embryonic Expression of Drosophila Semaphorins

Whole-mount dissections of stage 16 Drosophila embryos (anterior to left) showing expression of (A and B) *semaI* and (C–E) *semaII* as revealed by digoxigenin whole-mount in situ hybridization with gene-specific probes (A–E) and β-gal expression of the enhancer trap P element (P2) in the *semaII* gene.

(A and B) *semaI* RNA is expressed at high levels in a large subset of neurons in the CNS and at modest levels in some peripheral sensory neurons (black arrow in [A]).

(C–E) *semaII* RNA is expressed in a smaller subset of CNS neurons at high levels in a single T3 muscle (black arrow in C–E) and in the gonad (black arrowhead in C).

In (C), the darker staining at the anterior end of the embryo represents certain anterior sensory structures.

(E) At higher magnification, the *semaII* RNA is seen in the cytoplasm surrounding the nuclei in a single large T3 muscle (black arrow). As a landmark, the open arrowhead points to muscle 13 in the A1 segment (see Figure 5).

(F) β-Gal, driven by the P2 enhancer trap insert in *semaII*, is expressed in the nuclei of this same T3 muscle. Scale bar: (A and C) 30 μm; (B and D) 15 μm; (E and F) 7.5 μm.

in *semaII*^{P1} is nearly identical to that in *semaII*^{P2}, but is weaker in intensity.

The chromosomes containing each of these P element insertions in *semaII* contain other nonassociated lethal mutations (but not other P elements). Numerous mobilizations, many of which should result in the precise excision of the P elements, do not revert the homozygous lethality seen in each line (which should happen if the P element is the only source of lethality). In contrast, when examined as transheterozygotes over each other, or over deficien-

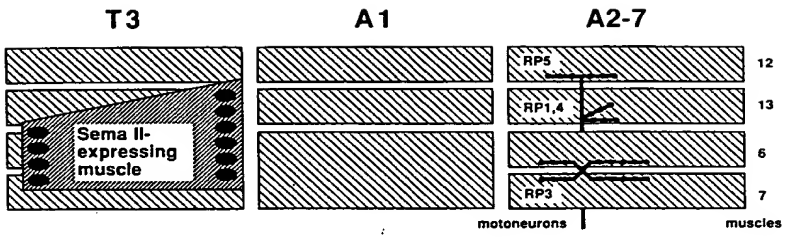


Figure 5. A Single Embryonic Thoracic Muscle Expresses Semaphorin II

Schematic diagram showing the location and identity of the embryonic ventral internal muscles in the T3, A1, and A2–7 segments. Much is known about the development of these muscles (7, 6, 13, and 12) and the motoneurons (RP3, RP1, RP4, and RP5) that innervate them in the A2–7 segments (see Broadie et al., 1993). Much less is known about the muscles in these same relative positions and the motoneurons that innervate them in the thoracic A1 segments. In the A1 segment, muscles 6 and 7 appear to be fused into a single muscle. In the T3 segment, these two muscles appear to be separate. However, there is an additional internal muscle that spans over muscles 7, 6, and 13. This T3-specific muscle expresses *semaII* at very high levels, as seen by both in situ hybridization (Figure 4E) and β-gal expression driven by an enhancer trap P element insertion in the *semaII* gene (Figure 4F).

cies made by the imprecise excision of either P element (see below), they show a consistent phenotypic profile that defines a loss-of-function condition for the *semaII* gene: greatly reduced eclosion, flightlessness and other behavioral defects, and death 2 days after adult eclosion.

semaII^{P1} over *semaII*^{P2} leads to the phenotypes outlined above. Precise excisions (defined genetically) of either one of these P elements, when placed in trans over the other insert, lead to 100% viability and a complete reversion of all phenotypes. *semaII*^{P1}, in which the P element is inserted in codon 32, is certainly a null mutation, but based on position alone, we do not know whether *semaII*^{P2} is a null or only a strong loss-of-function mutation. What argues for *semaII*^{P2} also being a null mutation is that imprecise excisions of this P element, which should lead to small deficiencies, display the same adult lethality when placed in trans over *semaII*^{P1}. Moreover, just as the precise excisions of the two inserts in trans lead to complete viability and reversion of all phenotypes, so the imprecise excisions of the two inserts in trans (using three different excisions of each element) lead to the same phenotypes as do the original inserts (see Experimental Procedures).

In *semaII*^{P1}/*semaII*^{P2}, only 24% of the expected number of mutants (n = 63) eclose as adult flies (the same pattern is seen in the imprecise excisions, whereas 100% eclose is seen in the precise excisions). Within 48 hr, all of the *semaII*^{P1}/*semaII*^{P2} mutant adult flies died (the same pattern is seen in the imprecise excisions, whereas <1% of the flies died within 48 hr in the precise excisions). The cause of death is unknown.

The adult mutant flies are completely flightless (n > 200). After they eclose, these flies have normal wings, which th y hold in the normal resting posture; however, shortly before dying, many hold their wings up. They can walk and jump, but they do not fly, even after a jump. When dropped from 0.5 m, they do not fly.

Th *semaII* mutant adult flies are also abnormal in a visual orientation test, even though they are normal in a phototaxis test, showing that they are not blind. When otherwise wild-type flies that are flightless (e.g., those flies that carry the CyO balancer chromosome to prevent them from flying) are placed in the center of a 40 cm arena that is surrounded by a circular 24 cm screen that is half black and half white, they typically walk to the edge of the arena within a few seconds at particular locations relative to the black and white (Wehner, 1972). Control (CyO) flies (within one day of eclosion) walk to the black portion of the screen 65% of the time, to the boundary between black and white 20% of the time, to the white portion of the screen 12% of the time, and they failed to walk to the edge within 30 sec in only 3% of the trials (n = 100).

In contrast, *semaII* mutant adult flies (*semaII*^{P1}/*semaII*^{P2}) (within one day of eclosion) showed a different profile of responses. A few mutant flies did not walk at all in the arena and were eliminated. Most of the *semaII* mutant flies did walk, and of these, 48% did not reach the edge within 30 sec; many of them instead walked in circles. The remaining 52% of the mutant flies did walk to the edge, 22% went to the black screen, 27% went to the boundary between black and white, and 3% went to the white (n = 100). Thus, nearly half of the *semaII* mutant flies did not properly visually orient and walk towards the edge of the arena, and of those that did walk to the edge, many did not show the normal preferences.

The *semaII* mutant flies appear abnormal in other behaviors as well. Adult wild-type flies will drink from a paper towel saturated with a 5% sucrose solution; when 0.4% bromophenol blue is added to this solution, the gut of wild-type flies turns blue (132 out of 134 adult flies turned blue). In contrast, the *semaII* mutant flies extend their mouth parts towards the sucrose solution, but 60% of their abdomens do not turn blue (only 49 out of 119 adult mutant flies turned blue), suggesting that some part of their drinking behavior is abnormal.

We analyzed the structure of the brains, heads, compound eyes, and thoracic muscles in 1 μm plastic sections of mutant adult flies, before their death, to determine if we could detect any gross abnormalities (n = 3). At this level of analysis, in the mutants we detect the normal number and position of neuronal cell bodies, the normal size and shap of the brain neuropils, the absence of any degenerative holes or other structural abnormalities in the brain or optic lobes, and the the normal number, size, and shape of the flight muscles. We also used a variety of antibodies (monoclonal antibody 102 to stain all CNS axons, monoclonal antibody 1D4 to stain a subset of longitudinal CNS axon tracts and all peripheral motor axons, and monoclonal antibody 22C10 to stain a subset of CNS axons and all peripheral sensory axons) to examine the overall shape and structure of CNS and peripheral axon path-

ways. We detected no gross abnormalities in the overall patterning of these axon pathways in *semaII* mutant embryos.

Discussion

In this paper, we define the Semaphorin family of transmembrane and secreted proteins that are conserved from insects to human. Two members of this family (G-Sema I in vivo [Kolodkin et al., 1992] and chick collapsin in vitro [Luo et al., 1993]) function in growth cone guidance. Mutations that reduce the function of a third in vivo (D-Sema II) result in abnormal adult behaviors, suggesting that it may be required for the construction of appropriate neural circuits. Including the signal sequence, the six eukaryotic Semaphorins range in size from 711 to 772 amino acids and are defined by a conserved 500 amino acid extracellular sema domain comprising two-thirds of each protein, and they contain 14–16 cysteines (thus far, 16 in insects and 14 of these 16 in vertebrates), many blocks of conserved residues, and no obvious repeats.

The first member of the family was G-Sema I (formerly Fas IV; Kolodkin et al., 1992). In the present paper, we describe the cloning and characterization of the probable homologs of this protein in two distant insect species (Tribolium and Drosophila) and of two additional members of the family (D-Sema II and H-Sema III). The sequence of a highly related protein (chick collapsin) was recently published (Luo et al., 1993) and, as described below, is likely to be the homolog of H-Sema III. In addition, we have also found or assembled two viral sequences (one from vaccinia and the other from variola virus) that encode truncated and more divergent Semaphorins.

Semaphorin I Function during Growth Cone Guidance in the Grasshopper

Although G-Sema I was initially isolated because of its expression on a subset of CNS axons, its expression on a stripe of epithelial cells in the limb bud has been the most accessible for functional analysis (Figure 1) (Kolodkin et al., 1992). The distal boundary of this Sema I stripe corresponds to the location where the pair of Ti1 growth cones make a sharp turn from proximal to ventral extension (O'Connor et al., 1990); as they turn, they extend along the distal portion of the Sema I stripe. They then make a second turn and extend proximally across the stripe following filopodial contact with the Cx1 neurons. When antibodies that block Sema I are added, the Ti1 growth cones often extend across this boundary, ultimately curving ventrally and contacting the Cx1 neurons, but in a more variable manner, at a more proximal location, and in a more highly defasciculated fashion, with each growth cone branching into multiple axons (Figure 1B).

These results show that sema I functions during growth cone guidance in vivo, suggest that it functions in the context of multiple competing guidance cues, but does not resolve how it functions. Sema I might function as an attractive cue, consistent with the turn and extension along the Sema I–expressing cells, but this model seems inconsistent with the change in fasciculation and branching seen in blocking experiments. S ma I might function as a repulsive cue, consistent with the general location of the turn and the changes in branching and fasciculation, but this model seems inconsistent (in the simplest notion of repulsion) with the ventral extension along the distal portion of Sema I–expressing cells (and with their ultimate extension across the Sema I stripe, although this could be explained by desensitization). Finally, Sema I might be a relative inhibitor (rather than an absolute repulsiv barrier) within the context of multiple guidance cues, inhibiting the response to the proximal cue and shifting guidance towards the ventral cue. This model is consistent with the location of the turn, the changes in branching and fasciculation in normal versus blocking experiments, and the ultimate extension over the Sema I stripe by overriding cues, and it is not inconsistent with extension along the distal portion of the Sema I stripe. The data seem more consistent with this model.

Comparison of Insect and Human Semaphorins with Chick Collapsin

While our work was being prepared for publication, th sequence of another member of the family (chick collapsin) was published (Luo et al., 1993). Collapsin prot in was purified from chick brain membrane extracts on the basis of its ability to promote the collapse of sensory growth cones in cell culture (Raper and Kapfhammer, 1990). It is unknown how it functions in the developing organism. Although purified as a membrane-associated protein, when expressed in COS cells, collapsin is secreted. Collapsin has an extracellular domain with significant sequence similarity to all of the Semaphorins. Of the 772 amino acids in collapsin, 520 (from amino acid 48 to 567, with 14 of 16 conserved cysteines) have significant sequence similarity to all of the Sema domains described here (Figure 2; Figure 3). All of the Semaphorins begin with a variable region of 30–60 amino acids that includes the signal sequence, followed by this conserved 500 amino acid Sema domain comprising over two-thirds of each protein. The transmembrane proteins (G-Sema I and its putative homologs T-Sema I and D-Sema I) then have an additional 80 amino acid stretch, a transmembrane domain, and a 80–110 amino acid cytoplasmic domain. Th secreted proteins (collapsin, D-Sema II, and H-Sema III) have an additional stretch of less than 20 amino acids, followed by a single Ig domain, and a 70 amino acid C-terminal region. Collapsin and H-Sema III, but not D-Sema II, then have a 45 amino acid basic domain.

In the Sema domain, chick collapsin and H-Sema III share 93% amino acid identity; this coupled with other similarities suggest that they are likely to be homologs. Of the two Drosophila proteins, D-Sema II is structurally most related to collapsin and H-Sema III, missing only the short C-terminal basic domain. However, in the Sema domain, collapsin shar s 35% identity with G-Sema I, 36% with D-Sema I, and 33% with D-Sema II, making it difficult to assign precise homologies amongst these insect and vertebrate molecules. Nevertheless, the structural and sequence similarity of D-Sema II with chick collapsin (and its

putative human homolog H-Sema III) suggests that these highly related proteins evolved from a common ancestral molecule which predated the split in the two lines leading to the arthropods and chordates.

Mutations in the *semall* Gene Lead to Behavioral Defects and Death

Loss-of-function mutations in the Drosophila *semall* gene lead to a set of phenotypes that includes reduced adult eclosion, a variety of adult behavioral abnormalities, and adult death within two days of eclosion. The most striking of these behavioral abnormalities is their complete inability to fly, even though they walk, jump, and have a flight apparatus (wings, wing posture, thoracic cuticle, and flight muscles) that appears normal. They are also abnormal in a visual orientation test (although they are not blind), and many are abnormal in their drinking behavior.

These behavioral abnormalities may reflect defects in underlying neural circuits. Alternatively, the abnormal behaviors might reflect defects in the muscles controlling th se behaviors, but superficially their flight muscles, for example, appear normal. At the gross structural level, we detect no major abnormalities in the patterning of CNS and peripheral nervous system axon tracts in the embryo, nor in the overall patterning of the brain neuropil in the adult. Thus, Sema II probably does not function in the overall patterning of axon pathways, but rather is likely to control the development of neural circuits, either by functioning in growth cone guidance or the formation of specific synaptic connections.

Semaphorin II Expression during Development f Neuromuscular Specificity

semall is expressed at its highest level by a single ventral muscle in the T3 segment during the period of its innervation. Much is known about the specific pattern of innervation of the abdominal ventral muscles (Figure 5) (reviewed by Broadie et al., 1993). In the T3 segment, another muscle develops just internal to these muscles, and this muscle alone expresses high levels of *semall* (Figures 4E–4F). Given its expression, Sema II might function to control the specificity of innervation of this muscle. The in vitro results on collapsin and the in vivo results on G-Sema I lead to a model whereby Sema II might function as a repulsive or inhibitory molecule that prevents neighboring ventral motoneurons from innervating this extra thoracic muscle. Alternatively, Sema II might function in an attractive fashion for the specific innervation of this muscle. It should be possible to determine which motoneurons innervate this Sema II–expressing muscle and how they and neighboring motoneurons behave in *semall* mutants. Thus, this muscle should allow us to test whether Sema II functions in synaptic specificity, and if so, whether in the organism it functions in an attractive or repulsive fashion.

Neural Semaph rins and the Growth Cone Resp nse

Of the six eukaryotic Semaphorins known, two function in growth cone guidance and a third is implicated by genetic analysis as functioning in the construction of behaviorally appropriate neural circuits. Chick collapsin functions in vitro to cause the collapse of sensory growth cones (Luo et al., 1993). G-Sema I functions in vivo to control the guidance of a pair of pioneering growth cones in the developing limb bud (Kolodkin et al., 1992) in a fashion consistent with its having some sort of inhibitory function. Finally, *semall* mutant adult flies are behaviorally abnormal, suggesting defects in underlying neural circuits. These results suggest that Semaphorins function in the organism in pathway or target recognition. Whether all Semaphorins function as repulsive or inhibitory guidance cues is not known, nor is it known whether any Semaphorin functions in this fashion in the organism in any or every context. One conclusion that emerges from the initial study (Kolodkin et al., 1992) and these recent studies (Luo et al., 1993; our paper) is that there must be growth cone receptors for the Semaphorins.

Viral Semaphorins and the Immune Response

The poxviruses are amongst the most complex viruses; they encode several hundred proteins. Recent studies have shown that some of these viral proteins appear to function in blunting the host inflammatory and immune defenses against viral infection (reviewed by Buller and Palumbo, 1991; Gooding, 1992). When secreted by the infected cell, some of these proteins inhibit known cytokines. For example, one sequence encodes a soluble form of the receptor for tumor necrosis factor (Smith et al., 1990), while another encodes a soluble receptor for γ-interferon (Upton et al., 1992). In many cases, the virus causes the infected cell to make a secreted protein that blocks the immune response of the host by either binding to a cytokine (as a dominant negative) or preventing activation of a cytokine (see Ray et al., 1992).

In this light, it is interesting to speculate on the function of the Semaphorins encoded by sequences found in both vaccinia (closely related to cowpox) and variola (human smallpox) viruses. The Sema proteins encoded by these two viruses are highly conserved with 92% amino acid identity. This Semaphorin is among the more highly conserved proteins encoded by these two viruses (Shchelkunov et al., 1993).

We suggest that these secreted, truncated Semaphorins are part of a mechanism used by these poxviruses to elude the immune response of the host. In contrast with making secreted receptors to remove positive cytokines, in the case of the Semaphorins the viruses appear to cause the infected cells to make a secreted form of the ligand. Thus, Semaphorins may function in the immune system as natural immunosuppressants to turn down the immune response. The two encoded by the poxviruses are highly divergent from the nervous system Semaphorins, and yet these differences, including both specific amino acids and inserted and deleted blocks of sequence, are completely conserved between the two viruses. The viruses may be mimicking a particular subfamily of Semaphorins that function and bind to specific receptors in the immune system.

Experimental Procedures

PCR Methods

Genomic DNA from Tribolium confusum (DNA from R. Denell) and

from Manduca sexta (embryos from J. Truman) were used at 1 μg and 40 ng per 100 μl PCR reaction, respectively. PCR was performed using Taq polymerase (Saiki et al., 1988) and the partially degenerate oligonucleotides GA[A/G]GA[C/T]GA[C/T]TG[C/T]CAGAA[C/T]TA (corresponding to G-Sema I amino acid sequence 104–110, EDDCQNY), and CC[A/G]CA[A/G]TT[A/G/T]AT[A/G]TA[C/T]TC[A/C/G]AC (corresponding to G-Sema I amino acids 227–233, VEYINCG). The PCR cycling conditions were as follows: three cycles of 94°C for 15 sec, 49°C for 30 sec, 49°C–72°C ramp for 2 min, and 72°C for 1.5 min, followed by 36 cycles of 94°C for 15 sec, 52°C for 1 min, and 72°C for 1.5 min; and finally followed by one cycle of 72°C for 10 min. These reaction products (450 bp from Tribolium and 557 bp from Manduca) were cloned into the SmaI site of M13mp10 and sequenced via dideoxy chain termination method (Sanger et al., 1977) and Sequenase (US Biochemical), as were all amplification products referred to below. Amplification products from both Tribolium and Manduca had small introns at different locations.

Drosophila Sema sequences were isolated as follows: cDNA to poly(A)+ RNA from Drosophila embryos at 6–15 hrs (25°C) of embryonic development was prepared and used at 20 ng per 100 μl PCR reaction. PCR was performed under the same conditions as those described above, using partially degenerate oligonucleotides. These were as follows: primer 1, GA[C/T]GA[C/T]TG[C/T]CA[A/G]AA[C/T]TA[C/T]AT (corresponding to G-Sema I amino acids 105–111, DDCQNYI); primer 2, [G/A]TA[G/A]TCNACNGCNGT[G/A]TC (corresponding to G-sema I amino acids 224–230, ETAVEY[I/M]); primer 3, CCNAGNCCNGGNCA[A/G]TG[C/T]GT (corresponding to G-Sema I amino acids 353–359, PRPGQCV); and primer 4, TCNAC[A/C]TT[A/G]TCCCANGC[A/G]CA[A/G]TA (corresponding to G-Sema I amino acids 511–518, YCAWDNVE). Primers 1 and 2 gave a product of 375 bp, primers 3 and 4 gave a product of 460 bp, and primers 1 and 4 gave a product of 1270 bp.

Human Sema sequence was isolated as follows: DNA isolated from 1×10^6 clones plated from a human fetal brain cDNA library (Stratagene) was used at 200 ng per 100 μl PCR reaction. PCR was performed under the same conditions as those described above, using partially degenerate oligonucleotides, which resulted in the amplification of a 830 bp product. These were as follows: TT[T/C]TT[T/C]TT[T/C]CGNGA[A/G]ACNGC (corresponding to G-Sema I amino acids 220–226, FFFRETA), and [A/G/T]AT[T/C]TC[A/G]TT[A/G]AA[A/G]TA[A/G]AANGG (corresponding to G-Sema I amino acids 509–515, DPYCAWD).

cDNA Isolation and Sequence Analysis

PCR products were used to screen 1×10^6 clones from appropriate cDNA libraries. cDNA clones were sequenced on both strands, and the deduced protein sequences were analyzed as previously described (Kolodkin et al., 1992).

T-semaI was isolated as follows. Clones (9) were recovered from a Tribolium embryonic λgt11 cDNA library (from R. Denell), and a single 2450 bp clone was sequenced. This cDNA has 254 bp of 5′ and 12 bp of 3′ untranslated sequences.

D-semaI was isolated as follows. Clones (40) were recovered from a Drosophila embryonic λgt11 cDNA library (Zinn et al., 1988), and two clones, 3050 bp and 3150 bp long, were sequenced. The 5′ ends of several other cDNA clones were sequenced as well. None of these clones encodes the N-terminal portion of the D-Sema I ORF. The two clones sequenced in their entirety contain 237 bp of 3′ untranslated sequence; they then diverge for the remainder of the 3′ untranslated sequence at what is a potential alternative splice site. One of these cDNA clones appears to be alternatively spliced and is missing 5 amino acids (GPQTSA; at relative position amino acid 596 of G-Sema I).

D-semaII was isolated as follows. cDNA clones (35) were recovered from a Drosophila embryonic λZAP cDNA library (from K. Zinn). One cDNA clone was sequenced in its entirety (containing the entire D-Sema II ORF), and 5′ and 3′ ends of several clones were sequenced using double-strand sequencing. These cDNA clones contain 267 bp of 5′ untranslated sequences and 222 bp of 3′ untranslated sequences.

H-semaIII was isolated as follows. cDNA clones (5) were recovered form a human fetal brain cDNA library in λZAP II (Stratagene). One clone was sequenced in its entirety and contains the complete H-Sema III ORF, 6 bp of 5′, and 264 bp of 3′ untranslated sequences.

Vaccinia and variola virus *semaIV* was identified as follows. Both viral sequences were identified by searches of the PIR data base using other Semaphorin sequences. For variola, only one of the reported ORFs, A43R, translates to encode part of a Sema domain. We thought this partial domain might be incomplete because it retained 92% amino acid identity with the vaccinia virus sequence in this region, and yet it had no signal sequence. We searched the nucleotide sequence to either side of the A43R ORF and were able to assemble the entire sequence with just a few frame shifts. This composite variola ORF is 92% identical at the amino acid level to the vaccinia Sema over its entirety, with no deletions or additions.

In Situ Hybridization and Immunocytochemistry

β-Gal was localized in embryos with an enhancer trap P element insertion (*semaII*^{P2}) in the *semaII* gene; the β-gal was visualized by staining the embryos with an anti-β-gal rabbit polyclonal antibody (from R. Holmgren), as previously described (Grenningloh et al., 1991). For in situ hybridization to Drosophila embryos, single-stranded sense and anti-sense RNA probes labeled with digoxigenin-11-dUTP (Boehringer Mannheim) were made from *semaI* and *semaII* cDNAs using T3 or T7 RNA polymerase, hybridized to Drosophila embryos in whole mount, and visualized (Kopczynski and Muskavitch, 1992) with minor modifications to increase sensitivity (C. Kopczynzki, personal communication).

Molecular and Genetic Analysis of P Element Insertions in the *D-semaII* Gene

The P element insertions in the *semaII* gene, *semaII*^{P1} and *semaII*^{P2}, were obtained from the Drosophila Genome Center and correspond to l(2)3021/CyO and l(2)3474/CyO. These lines contain the P element called PZ that includes the Escherichia coli *lacZ* reporter gene that drives the expression of β-gal into the nucleus (Mlodzik et al., 1990). *semaI* and *semaII* cDNA fragments labeled with bio-16-dUTP (Enzo Biochemicals) were used for in situ analysis of polytene chromosomes. For inverse PCR analysis of sequences flanking *semaIIP1*, genomic DNA was prepared from this line, and 0.5 μg aliquots were digested with several restriction enzymes. The digested DNA aliquots were ligated under dilute conditions and were then used as templates for PCR using primers (oriented away from each other) that are derived from the 3′ end of the P element. A DNA template generated with the enzyme SspI was used to amplify a single 750 bp product that was subcloned, sequenced, and found to contain a 3′ P element sequence that disrupts the sema II ORF in the middle of the codon (after the second bp) for amino acid 32. We used primers located in the 5′ end of the P element (facing out) and a position 200 bp downstream of the beginning of our cDNA clone (facing 5′) to amplify an 800 bp genomic fragment from the *semaII*^{P2} insertion line. For confirmation, we showed that this fragment hybridized with the *semaII* cDNA. Thus, the *semaII*^{P2} insertion is located 600 bp upstream from the start of our cDNA, which is likely not to include a significant portion of the complete 5′ untranslated sequences (on Northern blot analysis, the *semaII* mRNA is 4.0 kb, whereas our cDNA is 2.4 kb.)

Excisions of *semaII*^{P1} and *semaII*^{P2} were generated by crossing them to flies carrying a stable source of transposase activity (Robertson et al., 1988). We generated 47 excision lines of the *semaII*^{P1} insertion and 37 excision lines of the *semaII*^{P2} insertion. (We call the excision lines *semaII*$^{P1.n}$ and *semaII*$^{P2.n}$.) The phenotype of *semaII*^{P1} over 10 of the *semaII*$^{P2.n}$ excision lines gave the same phenotypic profile as *semaII*^{P1} over *semaII*^{P2} (see results), whereas *semaII*^{P1} over the other 27 *semaII*$^{P2.n}$ excision lines gave 100% viability and a complete reversion of all phenotypes. Thus, 10 of the *semaII*$^{P2.n}$ excision lines appear to define a loss-of-function condition, whereas the other 27 excision lines appear to be wild-type revertants. Similarly, the phenotype of *semaII*^{P2} over 39 of the *semaII*$^{P1.n}$ excision lines gave the complete mutant phenotypic profile, whereas *semaII*^{P2} over the other 8 *semaII*$^{P1.n}$ excision lines gave 100% viability and a complete reversion of the phenotypes, suggesting that these 8 lines were wild-type revertants. None of the imprecise P element excisions of either the *semaII*^{P1} or *semaII*^{P2} insertions gave phenotypes that were qualitatively different from the initial insertions when transheterozygous, suggesting that the initial insertions define the loss-of-function condition for the *semaII* gene.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are as follows: T-Sema I, L26080; H-Sema III, L26081; D-Sema I, L26082; and D-Sema II, L26083.